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Neurotrophic immunophilin ligands stimulate structural and functional recovery in neurodegenerative animal models

(calcineurin/cyclosporin A/cyclophilin/FK506/dopamine)

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ABSTRACT **Although immunosuppressant immunophilin ligands promote neurite outgrowth *in vitro*, their neurotrophic activities are clearly independent of their immunosuppressive activity. In the present report, a novel nonimmunosuppressive immunophilin ligand, GPI-1046 (3-(3-pyridyl)-1-propyl (2S)-1-(3,3-dimethyl-1,2-dioxopentyl)-2-pyrrolidinecarboxylate) is described. *In vitro*, GPI-1046 bound to FK506 binding protein-12 and elicited neurite outgrowth from sensory neuronal cultures with picomolar potency with maximal effects comparable to nerve growth factor. *In vivo*, GPI-1046 stimulated the regeneration of lesioned sciatic nerve axons and myelin levels. In the central nervous system, GPI-1046 promoted protection and/or sprouting of serotonin-containing nerve fibers in somatosensory cortex following parachloroamphetamine treatment. GPI-1046 also induced regenerative sprouting from spared nigrostriatal dopaminergic neurons following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine toxicity in mice or 6-hydroxydopamine (6-OHDA) toxicity in rats. The rotational abnormality in 6-OHDA treated rats was alleviated by GPI-1046. These neurotrophic actions in multiple models suggest therapeutic utility for GPI-1046 in neurodegenerative diseases.**

The immunosuppressant actions of drugs such as cyclosporin A and FK506 result from binding to receptor proteins, designated immunophilins, cyclophilin for cyclosporin A, and FK506 binding protein (FKBP) for FK506 (1). The drug-immunophilin complex binds to the calcium-activated phosphatase calcineurin, inhibiting its activity and resulting in accumulation of phosphorylated calcineurin substrates (2). One of these substrates, NFAT (nuclear factor of activated T cells), can enter the nucleus to stimulate interleukin 2 formation only in the nonphosphorylated state, so that drug treatment prevents its transcription factor activity, leading to immunosuppression (1, 3). Immunophilins also possess peptidyl prolyl cis-trans isomerase or rotamase activity, which facilitates protein folding and which is inhibited by immunosuppressant drug binding (2, 4–6). Inhibition of rotamase activity is not required for immunosuppression, because numerous drugs can bind to immunophilins and inhibit rotamase activity, but are not immunosuppressant because the drug-immunophilin complex fails to bind to calcineurin (3, 7).

The immunophilins are 10- to 50-fold more abundant in the nervous system than in immune tissues, and FKBP-12 is highly enriched in peripheral nerves (8, 9). Lesions of the facial or sciatic nerve lead to pronounced enhancement of mRNA levels of FKBP-12 in the facial and lumbar nuclei, respectively, and parallel an augmentation of growth-associated protein of 43 kDa (GAP-43) mRNA (9). Based on the association of GAP-43 with neurite extension, we evaluated the effects of immunosuppressant drugs upon neuronal outgrowth and observed potent augmentation of neurite extension from both cultured PC-12 cells and rat sensory ganglia explants (10). Low doses of FK506 *in vivo* were subsequently shown to augment physical regrowth and functional recovery of damaged sciatic nerves (11, 12).

Recently, we observed that nonimmunosuppressant as well as immunosuppressive immunophilin ligands are extremely potent in augmenting neurite outgrowth in sensory ganglia and PC-12 cells and enhancing morphologic and functional recovery in rats with damaged sciatic nerves (J.P.S., M.A.C., H.L.V., G.S.H., T. M. Dawson, L. Hester, & S. H. Snyder, unpublished work). Based on these observations, we synthesized a series of nonimmunosuppressive ligands of FKBP-12 and now report that one of these, 3-(3-pyridyl)-1-propyl (2S)-1-(3,3-dimethyl-1,2-dioxopentyl)-2-pyrrolidinedinecarboxylate (GPI-1046), is neurotrophic in multiple neuronal systems and efficacious in promoting both morphologic and functional recovery in rodent models of peripheral nerve injury and neurodegenerative disorders.

MATERIALS AND METHODS

***In Vitro* Studies.** Rotamase activity of FKBP-12 was assayed as described by Kofron (13), using the peptide *N*-succinyl Ala-Leu-Pro-Phe p-nitroanilide (Bachem) as substrate. Dorsal root ganglia were dissected from embryonic chick, gestation day E8–E10, and explants of sensory neurons were cultured as described (J.P.S., M.A.C., H.L.V., G.S.H., T. M. Dawson, L. Hester, and S. H. Snyder, unpublished work). Neurite outgrowth was assessed from photomicrographs of each explant culture and all processes whose length exceeded the explant's diameter were counted.

***In Vivo* Studies.** The sciatic nerve of adult male Sprague–Dawley rats (n = 6 per group) was lesioned. Rats received s.c. injections of GPI-1046 at 3 or 10 mg/kg in Intralipid vehicle (Clintec Nutrition, Deerfield, IL), and were processed to quantitate axonal caliber, cross-sectional area and myelin as described (J.P.S., *et al.*, unpublished work). Serotonin neurons of adult male Sprague–Dawley rats were lesioned with 10 mg/kg parachloroamphetamine (PCA; ref. 15). Animals (n =

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Abbreviations: GPI-1046, 3-(3-pyridyl)-1-propyl (2S)-1-(3,3-dimethyl-1,2-dioxopentyl)-2-pyrrolidinecarboxylate; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; 6-OHDA, 6-hydroxy dopamine; TH, tyrosine hydroxylase; PCA, parachloroamphetamine; FKBP, FK506 binding protein; GAP-43, growth-associated protein of 43 kDa.

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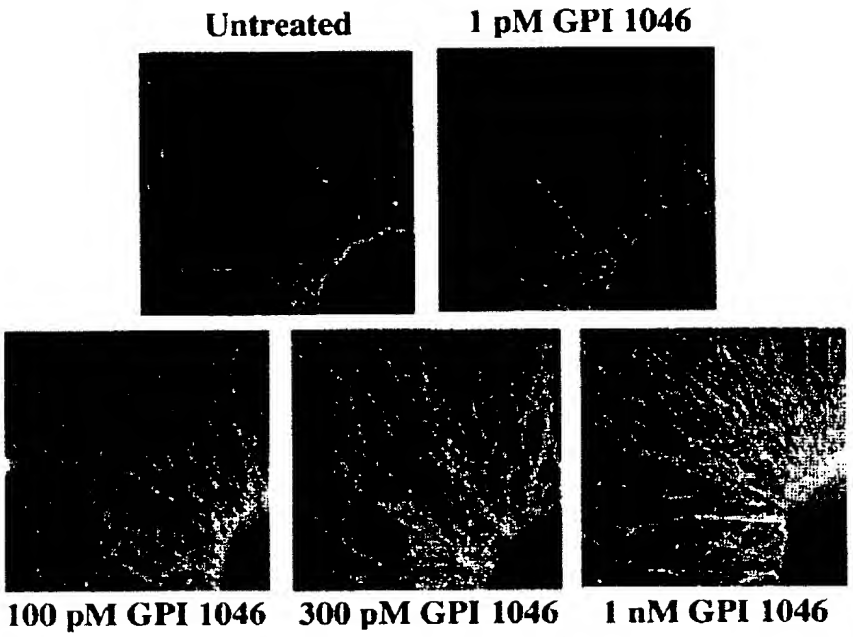


FIG. 1. GPI-1046 elicits neurite outgrowth in chicken sensory neuronal cultures. Increasing concentrations of GPI-1046 were added to sensory neuronal cultures, and neurite outgrowth (number of neurites whose length is larger than the diameter of the explant) at 48 hr posttreatment was quantitated.

6 per group) were treated with GPI-1046 at 40 mg/kg s.c. daily for 3 days before PCA treatment, and daily for 2 subsequent weeks. The density of serotonin-immunoreactive fibers in the somatosensory cortex of sham, lesioned and lesioned/GPI-1046-treated animals was quantitated. Rodent models of Parkinson disease were produced by damaging the nigrostriatal dopaminergic system. Male CD1 mice (20–25 g) received five daily i.p. injections of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, 30 mg/kg) in saline vehicle. Two different GPI-1046 dosing paradigms were used, a "concurrent" paradigm of daily s.c. GPI-1046 dosing 30 min before each MPTP injection and on each of 5 subsequent days, and a delayed "post-MPTP" paradigm of 5-day s.c. GPI-1046 treatment beginning 4 days after cessation of MPTP treatment (n = 10 animals per group). The animals were sacrificed 18 days after initiation of MPTP treatment. Male Sprague–Dawley rats (250–275 g) received unilateral 2.0-μl stereotaxic injections of 10 μg/ml 6-hydroxy dopamine (6-OHDA) in 0.04% ascorbic acid into the substantia nigra. The efficacy of GPI-1046 was examined in three studies. In the first study, GPI-1046 (10 mg/kg s.c.) was first given 1 hr after 6-OHDA, whereas rats in the second and third studies received their first GPI-1046 injections 7 days or 28 days after 6-OHDA treatment, respectively, in n = 8 animals per group. The GPI-1046 treatments continued for 5 days, and the animals were sacrificed 14 days after their final GPI-1046 injection. For each 6-OHDA experiment, 8 animals per treatment group were used for examination of rotation induced by s.c. administration of amphetamine (3 mg/kg) 2 weeks after the last GPI-1046 injection, just before sacrifice. Rotations were counted manually over a 1-hr period following drug administration. Striatal dopamine and metabolites were quantitated by electrochemical detection using a Hewlett–Packard model 1100 HPLC system. The density of striatal fibers and nigral neurons was determined using tyrosine hydroxylase (TH) immunohistochemistry. Blinded analysis of TH fiber density in the central striatum was performed at ×630 and the percentage of striatal fields covered by TH-positive processes and terminals was calculated

Table 1. GPI-1046 augments regrowth of damaged sciatic and central serotonin neurons

Treatment	Diameter, μm	Cross-sectional area, μm^2	Myelination	Serotonin innervation density
		Sciatic nerve crush		
Sham	3.462 ± 0.148	12.04 ± 0.949	63.44 ± 8.50	
Crush/vehicle	1.944 ± 0.071	4.606 ± 0.324	3.18 ± 1.59	
Crush/GPI-1046 (3 mg/kg)	2.656 ± 0.079*	8.169 ± 0.452*	21.66 ± 5.94*	
Crush/GPI-1046 (10 mg/kg)	2.742 ± 0.042*	8.866 ± 0.337*	24.31 ± 7.61*	
		PCA lesions of serotonin neurons		
Vehicle/vehicle				20.98 ± 0.70
PCA/vehicle				3.83 ± 0.26
PCA/GPI-1046 (40 mg/kg)				7.67 ± 0.78**

Animals lesioned by sciatic nerve crush at the level of the hip were treated by daily s.c. injections of GPI-1046 for 18 days, followed by sacrifice. Axonal diameter and cross-sectional area were assessed by quantitative analysis of anti-neurofilament stained sections of nerve. Axon diameter and cross-sectional area are expressed as mean ± SEM. The myelination levels are expressed as the mean ± SEM of myelin basic protein-immunoreactive stain density of 2–3 fields per section of each experimental group. The values for mean diameter, cross-sectional area, and myelination from lesioned animals treated with GPI-1046 are statistically different from crush/vehicle-treated animals. *, $P < 0.001$ by Student's t test. Results of the PCA lesions of serotonin neurons are expressed as serotonin-positive innervation density ± SEM of 2–3 fields per section of each animal per group. **, Statistically different from PCA/vehicle by Student's t test; $P < 0.05$.

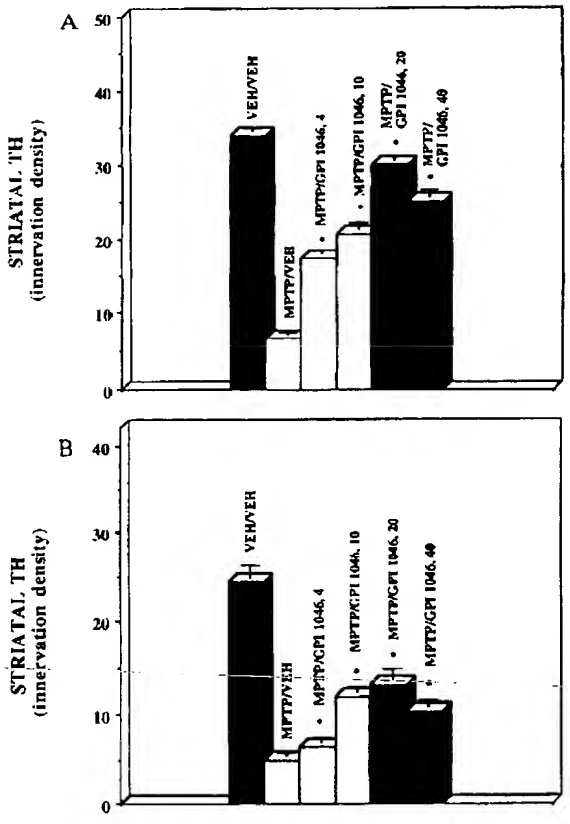
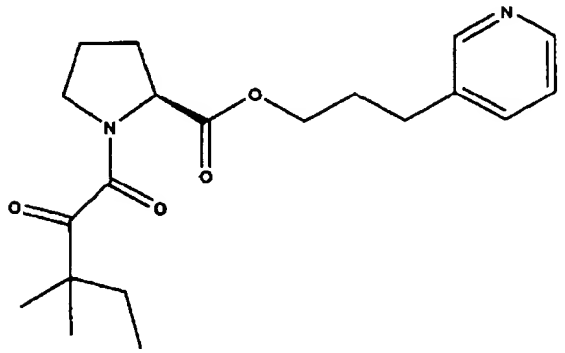


FIG. 2. GPI-1046 protects against loss of striatal TH and promotes regeneration of striatal dopaminergic markers in MPTP-treated mice. (*A*) Dose dependency of GPI-1046-mediated recovery of striatal TH innervation density in the concurrent MPTP-GPI-1046 model. Quantitative analysis of striatal TH levels was as described. At all dose levels, TH innervation density was significantly greater than MPTP/vehicle-treated cases alone (Student's *t* test, $P < 0.001$). (*B*) The dose-dependent recovery of TH+ labeled punctae is evident in the striata of MPTP-lesioned mice treated after an 8-day delay with s.c. GPI-1046 at 4, 10, 20, or 40 mg/kg. Quantitation of the post-MPTP-administered GPI-1046 in regeneration of striatal TH innervation density is depicted graphically. At all dose levels, TH innervation density was significantly greater than MPTP treated cases alone (Student's *t* test, $P < 0.001$).

using an image-analysis program (SIMPLE, Compix, Pittsburgh, PA). The total number and density of TH-positive nigral neurons were determined at ×250. All comparisons were evaluated using Student's *t* test.

RESULTS

Design and Synthesis of GPI-1046. Previous work by Schreiber (1) established that FK506 comprises two distinct binding domains. One portion of the molecule binds at the prolyl isomerase active site of FKBP-12. The remainder of the FK506 molecule forms part of the calcineurin-binding domain of the FK506/FKBP-12 complex, and has been termed the "effector" domain. Compounds capable of binding to the enzymatic active site of FKBP-12 but lacking an effector domain would be expected to function as nonimmunosuppressive FKBP-12 ligands. Using the principles of structure-based drug design, we synthesized a number of potent, selective small molecule inhibitors of FKBP-12 that are devoid of immunosuppressive activity but possess potent neurotrophic activity *in vitro* and *in vivo*. GPI-1046 is typical of this new class of small molecule neurotrophic drugs.



Scheme 1

GPI-1046 Stimulates Neurite Outgrowth from Sensory Ganglia. In the absence of exogenously added growth factors, GPI-1046 is extremely potent in augmenting neurite outgrowth in chicken sensory ganglia, with significant enhancement evident with as little as 1 pM concentration (Fig. 1). Maximal stimulation at 1–10 nM elicits outgrowth comparable to that observed with maximal concentrations of nerve growth factor. Fifty percent of maximal stimulation is evident at 58 pM.

GPI-1046 Is Nonimmunosuppressive. GPI-1046 was designed as a nonimmunosuppressive ligand of the immunophilin FKBP-12, and we tested its ability to act as an immunomodulator in T cell proliferation assays. GPI-1046 lacks immunosuppressive effects in concanavalin A-stimulated peripheral blood lymphocytes. At concentrations of up to 10 μM, GPI-1046 fails to inhibit cellular [^{3}H]thymidine incorporation, while FK506 and cyclosporin A in low nanomolar concentrations are inhibitory (data not shown). GPI-1046 (10 μM) also does not inhibit the phosphatase activity of purified calcineurin-FKBP-12 assayed with phosphorylated microtubule-associated protein (MAP-2, data not shown). Therefore, GPI-1046 is an FKBP-12 ligand with no immunosuppressive effects.

GPI-1046 Stimulates Recovery Following Sciatic Nerve Crush. FK506 (refs. 11 and 12; J.P.S., *et al.*, unpublished work) and a nonimmunosuppressant immunophilin ligand (J.P.S., *et al.*, unpublished work) augment recovery of damaged sciatic nerves. GPI-1046 also displays this activity when administered at 3 or 10 mg/kg s.c. daily beginning on the day of nerve crush until sacrifice 18 days later (Table 1). GPI-1046 treatment markedly augments both the diameter and cross-sectional area of the recovering nerve fibers, though at the 18-day time point recovery is not complete. Effects of GPI-1046 on myelin levels are even more striking, with 7- to 8-fold higher levels in drug-treated than vehicle-treated animals. In the vehicle-treated animals, microscopic myelin sheaths are not evident, whereas substantial myelin layering is seen in injured nerves after GPI-1046 treatment.

GPI-1046 Augments Serotonin Fiber Recovery Following Lesions with PCA. PCA produces marked destruction of central serotonin neurons (14). Rats received GPI-1046 for 3 days, at which time PCA was administered, followed by GPI-1046 for an additional 14 days when the animals were sacrificed. In rats treated with PCA, forebrain serotonin immunoreactivity is markedly reduced (Table 1). PCA-treated animals receiving GPI-1046 display double the density of serotonin positive neuronal fibers in the somatosensory cortex.

GPI-1046 Stimulates Recovery of the Nigrostriatal Dopamine System in Mouse Models of Parkinson Disease. MPTP destroys dopamine neurons following oxidation by monoamine

oxidase B to 1-methyl-4-phenylpyridinium ion (MPP^+), which is avidly accumulated by the dopamine transporter, and then destroys the neurons by oxidative free radical mechanisms (15). MPTP reduces the density of TH-positive striatal axonal processes by about 77% (Fig. 2*A*). GPI-1046 administered at 4 mg/kg in the concurrent dosing paradigm more than doubles the number of spared striatal TH-positive processes compared with MPTP/vehicle controls. Maximal protective effects in this model are evident at GPI-1046 doses of 20 mg/kg, which elicits a 4- to 5-fold increased density of striatal TH-positive fibers and a similar magnitude increase in the density of striatal fibers positive for dopamine transporter immunohistochemistry (data not shown). As the transporter is localized to membranes of dopaminergic fibers, the observed striatal reinnervation reflects an increase in the number of dopaminergic axonal processes and terminals and not a nonspecific upregulation of TH levels. Additionally, the depletion of dopamine and its metabolites following MPTP treatment is substantially prevented by GPI-1046 administration (data not shown).

To more faithfully model human Parkinson disease, we examined effects of GPI-1046 when administered in the post-MPTP paradigm, where GPI-1046 is not administered until maximal destruction of dopamine neurons has taken place. In this paradigm, GPI-1046 treatment greatly enhances striatal innervation density with significant augmentation evident at 4 mg/kg (Fig. 2*B*). Maximal effects are evident at 20 mg/kg with striatal innervation densities 2- to 3-fold higher than untreated MPTP/vehicle controls. Striatal reinnervation in GPI-1046-treated animals is characterized by many clusters or small branches of processes emerging from the sparse network of spared nigrostriatal fibers, suggestive of terminal and collateral sprouting.

GPI-1046 Stimulates Morphological, Biochemical, and Functional Recovery of the Rat Nigrostriatal System Following 6-OHDA Lesions. 6-OHDA is accumulated by dopamine neurons and oxidized to quinone, and it elicits neuronal destruction (16, 17). Intranigral 6-OHDA produces 90–95% depletion of TH immunoreactive fiber density in the striatum (F.g. 3). GPI-1046 treatment elicits a pronounced increase in striatal TH-positive fiber density of comparable magnitude when given 1 hr, 1 week, or 1 month following the lesion (Table 2). The density of striatal TH-positive fibers in GPI-1046-treated rats is 2.5–3.5 times greater than in 6-OHDA/vehicle-treated controls (Fig. 3).

Table 2. GPI-1046 enhances regrowth of 6-OHDA lesioned striatal dopamine neurons

Treatment	Striatal TH innervation density
Vehicle/vehicle	22.61 ± 1.78
6-OHDA/vehicle	3.06 ± 0.55
6-OHDA/GPI-1046, 1 hr	9.75 ± 1.92*
6-OHDA/GPI-1046, 7 days	8.04 ± 1.38*
6-OHDA/GPI-1046, 28 days	8.56 ± 1.59*

Rats were lesioned with 6-OHDA and treated with daily GPI-1046 (10 mg/kg, s.c.) beginning 7 days following the lesion. Animals were sacrificed 18 days after the lesion. Striatal TH levels were determined from 5 fields per section of each animal in the experimental group. *, Statistically different from 6-OHDA/vehicle-treated animals; $P < 0.05$.

Rats with unilateral 6-OHDA lesions of the substantia nigra rotate ipsilateral to the lesion when treated with amphetamine (18). The pronounced rotation observed with amphetamine in 6-OHDA-treated animals is significantly diminished in rats receiving 10 mg/kg GPI-1046 1 week following the 6-OHDA lesions (Table 3). In these animals, drug treatment restores striatal dopamine to ≈30% of control levels. The dramatic abolition of the functional deficit fits with abundant evidence that only about a third of normal dopamine innervation is required for physiologic motor activity.

DISCUSSION

One of the most striking features of the neurotrophic actions of immunophilin ligands is their extraordinary potency. Previously we reported picomolar neurotrophic effects of FK506 in PC-12 cells and sensory neuronal cultures (ref. 10; J.P.S., *et al.*, unpublished work). GPI-1046 produces significant enhancement of neurite outgrowth in sensory ganglia at 1 pM, with 50% of maximal stimulation at 58 pM. By contrast, its K_i for inhibiting the rotamase activity of FKBP-12 is ≈7.5 nM. Thus, the neurotrophic potency is about 100 times greater than the drug's apparent affinity for FKBP-12. FK506 is also more potent in stimulating neurite outgrowth than in binding to FKBP-12 and inhibiting its rotamase activity (ref. 10; J.P.S., *et*

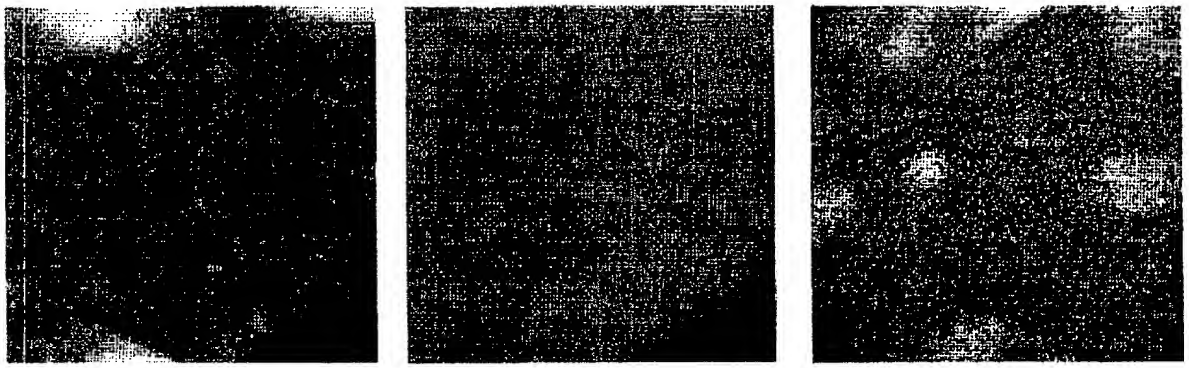


FIG. 3. GPI-1046 increases striatal TH+ innervation density after intranigral 6-OHDA injection. The dense pattern of TH+ punctae and processes that characterize the normal caudate putamen is evident in the caudate putamen contralateral to the 6-OHDA injection (*Left*). Extensive loss of TH+ punctae is evident in the striatum ipsilateral to the 6-OHDA injection (*Center*), but a sparse network of TH+ fibers are spared (*Right*). In cases treated with daily GPI-1046 (10 mg/kg, s.c.) for 5 days beginning 1 week after the 6-OHDA injection, an increase in the density of TH+ labeled fibers is evident together with an increase in the number of TH+ varicosities. Veh, vehicle.

Table 3. GPI-1046 reverses rotational motor abnormalities in 6-OHDA lesioned rats

Treatment	Duration of rotations, min	Rate of rotations, rotations/hr
Vehicle/vehicle	0	0
6-OHDA/vehicle	28.0 ± 8.0	192.0 ± 52.7
6-OHDA/GPI-1046	2.3 ± 1.5*	65.0 ± 15.5*

6-OHDA lesioned animals treated with GPI-1046 (10 mg/kg, s.c.) 1 week postlesion were evaluated for their response to amphetamine-induced rotation 14 days following their last GPI-1046 treatment. On day 11 after the last GPI-1046 treatment, the rats were given 3 mg/kg amphetamine as a sensitizing dose. On day 14 following GPI-1046, animals were treated with 3 mg/kg amphetamine, and their rotational behavior was quantitated by manual observation. The mean values ± SEM for duration of rotation and maximal rate of rotation are presented. *, Results are statistically significantly different from 6-OHDA/vehicle; $P < 0.05$.

al., unpublished work). Conceivably, the "receptor" immunophilin in chicken sensory ganglia is a different form of FKBP than FKBP-12 and has higher affinity for these drugs. Alternatively, the drugs may concentrate within the ganglia.

Previous studies demonstrated that the immunosuppressive immunophilin ligand FK506 stimulates functional as well as morphologic recovery following sciatic nerve damage (refs. 11 and 12; J.P.S., *et al.*, unpublished work). GPI-1046 stimulated regrowth of axons in the injured sciatic nerve and markedly enhanced their myelination, suggesting that multiple sclerosis may be a potential therapeutic target for GPI-1046.

GPI-1046 stimulated striatal reinnervation by dopamine fibers in models of Parkinson disease produced by MPTP in mice and 6-OHDA in rats. Consistent morphological recovery in these models was accompanied by biochemical recovery of dopamine levels and, in the rat 6-OHDA model, a dramatic recovery from motor abnormalities, suggesting therapeutic utility in Parkinson disease.

GPI-1046-mediated recovery of striatal dopaminergic markers in the concurrent MPTP dosing model is greater than that previously reported for agents that do not directly interfere with either conversion of MPTP to MPP^+, or uptake of MPP^+ into dopaminergic terminals (19). Unlike numerous compounds with demonstrated neuroprotective action in mouse MPTP models, GPI-1046 does not interfere with MPTP neurotoxicity by known mechanisms such as monoamine oxidase B inhibition, dopamine uptake inhibition, or excitatory amino acid antagonism (J.P.S., G.S.H., D.T.R., H.L.V., M.A.C., and P.D.S., unpublished observations). Recovery of striatal dopaminergic markers occurred even when GPI-1046 administration was delayed longer than 1 week after MPTP treatment onset, after the reported peak of nigral neuronal death (20, 21). Thus, the increased striatal innervation reflects sprouting of processes from residual axons and not the protection of nigral cell bodies. Regeneration of striatal dopaminergic markers in the post-MPTP dosing regimen with GPI-1046 was greater than that reported for epidermal growth factor (22, 23), nerve growth factor (24), glial cell line-derived neurotrophic factor (25), and gangliosides or their synthetic derivatives (19, 26, 27). Unlike polypeptide neurotrophic factors, which must be administered intracranially to elicit an effect, GPI-1046 induced significant dose-dependent striatal dopaminergic reinnervation in MPTP-treated mice following systemic s.c. administration. GPI-1046 stimulated dopaminergic axonal sprouting in the striatum when given as long as 1 month after 6-OHDA lesion. To our knowledge, no other trophic agent has been reported to be effective in such a model. In contrast, glial cell line-derived neurotrophic factor, the most efficacious polypeptide neurotrophic factor in the dopamine system studied to date, failed to stimulate striatal sprouting of residual dopaminergic axons in monkeys when administered 90 days following MPTP (28).

GPI-1046 also stimulated serotonin neuronal recovery following destruction by PCA (Table 1). Thus, unlike peptidic neurotrophic factors that display distinct neurotrophic activities in overlapping but limited central nervous system neuronal populations, immunophilin ligands are neurotrophic for diverse neural systems, including PC-12 cells and sensory ganglia *in vitro*. In intact animals, the drugs stimulate regrowth of damaged facial (data not shown) and sciatic nerves, and brain dopamine and serotonin neurons.

Unlike many other neurotrophic polypeptides, immunophilin ligands do not induce aberrant sprouting of neuronal processes when administered to normal animals. In normal rats and mice, we have carefully examined sciatic and facial nerves as well as numerous areas of the brain and spinal cord and failed to observe any suggestions of abnormal sprouting (data not shown). By contrast, nerve growth factor elicits sprouting of normal sensory neurons associated with hyperalgesia (29).

GPI-1046 displays excellent bioavailability, readily crosses the blood–brain barrier, and is active following oral administration (J.P.S., G.S.H., D.T.R., H.L.V., M.A.C., and P.D.S., unpublished work), whereas the clinical application of peptidic growth factors is hampered by limited bioavailability. Accordingly, GPI-1046 and related agents may find therapeutic application in various neurodegenerative disorders.

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